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Tumor Aneuploidy

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Introduction

The research in this proposal is designed to test the hypothesis that aneuploidy in some breast tumors is caused by centrosome abnormalities which are induced by alteration in p53 function. Specific mutations and expression alterations in p53 that are associated with breast cancer, aneuploidy, and centrosome abnormalities will be identified. In order to test whether or not a specific p53 mutation affects centrosome structure and function, primary cultures of normal mammary epithelial cells will be transfected to over-express the mutant p53. These transfected cells will then be monitored for changes in ploidy and centrosome structure and function. It is expected that significant novel information will emerge from these studies. First, tumor aneuploidy will be related to a spectrum of p53 mutations or expression alterations. Insights into the function of p53 will be gained by associating specific mutations with specific tumor phenotypes. An important aspect is that this portion of the research will be performed on human tumors rather than in animal models or established cell lines. Second, the effects of the mutations will be tested on primary cultures of normal human mammary epithelial cells. This portion of the research will actually test the hypothesis that some p53 mutations directly affect centrosome structure and function, resulting in aneuploidy. If specific p53 mutations lead to aneuploidy by affecting centrosomes, then the possibility arises for development of new therapies that target centrosome function. Cultures of primary mammary epithelial cells are being used instead of established cell lines, even though primary cells have a limited number of passages before they begin to senesce. The increased validity of results from non-immortalized primary cells warrants the technical challenge their use imposes.

Annual Summary

Research Accomplishments

As indicated in 1999 Progress Report, I revised my statement of work in order to use a new Mayo Facility for the p53 mutation screening portion of the project. Research Accomplishments to date reflect the Tasks as outlined in the revised Statements of Work.

Task 1 - Quantification of structural and functional centrosome alterations (months 1-12).

Approximately 26 tumor and 13 benign tissues have been analyzed for centrosome volume and 34 tumor and 13 benign tissue have been analyzed for MT nucleation capacity. Correlative light and electron microscopy analysis of 6 benign tissues and 28 tumors has revealed that tumors with centrosome abnormalities have higher proliferative, mitotic, and abnormal mitotic indices than do benign tissues or tumor tissues with normal centrosomes. **Interestingly, one specific centrosome abnormality, excess pericentriolar material, is associated with the highest frequency of abnormal mitoses. These data are included in a manuscript that has been published in the American Journal of Pathology (Appendix 3).** This Task is complete.

Task 2 - Screen tissues for aneuploidy (months 10-18). To date, approximately 35 benign and tumor tissues have been analyzed for ploidy using FISH analysis of chromosomes 3,7, and 17. **All benign tissues were diploid, 3 of 21 tumors were diploid or near diploid, while 18 were aneuploid. Tumor aneuploidy correlated nearly 100% with centrosome amplification. The**

diploid tumors had nearly normal centrosome characteristics. The methods in use have been changed from the original proposal to yield specific information on chromosome 17, which is the location of the p53 gene. This Task is near completion. Data will be included in a manuscript that is currently in preparation.

Task 3 – Trial site-directed mutagenesis and trial transfection. A p53 mutated from glycine to serine at amino acid 245 was selected based on its occurrence in Li-Fraumeni families having a high incidence of breast cancers. **Initial results indicated that cells transfected with the p53 mutant develop a phenotype consistent with the hypothesis, namely centrosome and mitotic spindle abnormalities are present at a much higher frequency in the presence of mutant p53 than they are in normal cells.** Because transfected primary mammary epithelial cells have proven too difficult to work with, we are now using primary mammary epithelial cells that have been transfected with human telomerase for these studies. These cells were obtained from Geron, Inc. under a Materials Transfer Agreement. Analysis of the transfectants is ongoing.

Task 4 – p53 mutation/immunohistochemistry status (months 16-30). All tissues have been analyzed for p53 immunohistochemistry. **Normal tissues had no significant p53 immunostaining. Benign tumors had an average value of 3.6% of the cells with p53 immunostaining (ranging from 0 to 10% of the tumor cells). Malignant tumors ranged from 0% (13 of 40 analyzed) to more than 75% (8 of 40 analyzed), with a mean of 13.8%.** DNA has been extracted from all tissues, and PCR amplification of the exons is nearly complete. Mutation screening is scheduled to be performed using DHPLC in July, 2000. Mutations identified by DHPLC will be confirmed by sequencing. This task is scheduled for completion by August, 2000. These data will be included in a manuscript that is currently in preparation.

Task 5 – Analysis of data from Tasks 1, 2, and 4 (months 31-33). This task is in progress.

Task 6 – Site-directed mutagenesis of p53 using mutant identified in Task 5 (months 33- 36). Not yet begun.

Task 7 – Transfection and monitoring experiments (months 35-46). Not yet begun.

Task 8 – Data analysis and manuscript preparation (months 38-48). The first paper resulting from this project has been published in American Journal of Pathology (155:1941-1951). Data from Tasks 1, 2, and 4 are being analyzed and a second manuscript is in preparation.

1) Key Research Accomplishments

- Excess pericentriolar material is a specific centrosome defect associated with an increased frequency of abnormal mitoses in human breast tumors.
- A specific p53 mutation (glycine to serine at amino acid 245) induces abnormal centrosome structure and function upon transfection of primary normal human mammary epithelial cells.
- **Aneuploidy has nearly 100% correlation with centrosome abnormalities in human breast tumors.**

2) Reportable Outcomes

- Seminar presented: GI Unit Scientific Meeting, Mayo Clinic. March 26, 1999. "Aberrant Structure and Function of Centrosomes in Human Breast Tumors".
- Promotion received: from Senior Research Fellow to Associate Consultant, effective March 1, 1999.
- **Paper published: "Altered Centrosome Function is Associated with Abnormal Mitoses in Human Breast Tumors" Lingle, WL and Salisbury, JL. American Journal of Pathology, 155:1941-1951. Cover Photograph. (See Appendix 3).**
- **Book Chapter Published: "The Role of the Centrosome in the Development of Malignant Tumors" Lingle, WL and Salisbury, JL. Current Topics in Developmental Biology, Vol 49:313-329. In Press. (See Appendix 4 Galley Proofs).**
- **Funding Applied for: DoD Breast Cancer Research Program 2000 Idea Award. "Investigation of Gene Expression Correlating wit Centrosome Amplification in Development and Progression of Breast Cancer".**

Appendices

See attached

Altered Centrosome Structure Is Associated with Abnormal Mitoses in Human Breast Tumors

Wilma L. Lingle and Jeffrey L. Salisbury

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Rochester, Minnesota

Centrosomes are the major microtubule organizing center in mammalian cells and establish the spindle poles during mitosis. Centrosome defects have been implicated in disease and tumor progression and have been associated with nullizygosity of the p53 tumor suppressor gene. In the present ultrastructural analysis of 31 human breast tumors, we found that centrosomes of most tumors had significant alterations compared to centrosomes of normal breast tissue. These alterations included 1) supernumerary centrioles, 2) excess pericentriolar material, 3) disrupted centriole barrel structure, 4) unincorporated microtubule complexes, 5) centrioles of unusual length, 6) centrioles functioning as ciliary basal bodies, and 7) mispositioned centrosomes. These alterations are associated with changes in cell polarity, changes in cell and tissue differentiation, and chromosome missegregation through multipolar mitoses. Significantly, the presence of excess pericentriolar material was associated with the highest frequency of abnormal mitoses. Centrosome abnormalities may confer a mutator phenotype to tumors, occasionally yielding cells with a selective advantage that emerge and thrive, thus leading the tumor to a more aggressive state. (*Am J Pathol* 1999, 155:1941-1951)

Checkpoints monitor the nuclear cycle and signal progression after proper completion of earlier stages of the cell cycle.¹ Differentiation, cell proliferation, and programmed cell death are normal outcomes of checkpoint surveillance. In cancer, dysregulation of the cell cycle can result in either a decrease in the rate of cell death or an increase in the rate of cell division, and thereby lead to tumor growth. The orderly duplication of the centrosome once, and only once, in each cell cycle and the formation of a bipolar mitotic spindle are key cell cycle checkpoints leading to successful cell division. The importance of the centrosome in the development of malignant tumors was suspected first by Boveri² nearly 100 years ago. More recently, centrosome defects have been implicated in disease and tumor progression.³⁻¹³ Defects in centrosome duplication, alteration in centrosome microtubule nucleation capacity, and inappropriate phosphorylation

of centrosome proteins were first described for human breast tumors¹⁴ and subsequently, centrosome anomalies were reported for other tumors.¹⁵⁻¹⁷ Recent evidence suggests that elevated Aurora kinase or Serine/Threonine kinase-15 (STK15) activity may play a key role in acquisition of at least some of these centrosome defects during tumor progression.¹⁸

The centrosome is the major microtubule-organizing center in mammalian cells; it regulates the number, stability, polarity, and spatial arrangement of microtubules in interphase cells.^{19,20} Thereby, the centrosome and microtubules play a role in maintaining overall cell polarity, provide an architectural framework for directed organelle transport, and participate in cell shape and movement.

The interphase centrosome consists of a pair of orthogonally oriented centrioles surrounded by a pericentriolar matrix. Duplication of the centrosome begins during S phase of the cell cycle when the two centrioles lose their orthogonal arrangement before the formation of a procentriole (or bud) closely associated with the proximal end of each of the original centrioles. The procentrioles lengthen during S and G₂, so that by prophase the cell contains two diposomes, that is, two orthogonal pairs of full-length centrioles.²¹⁻²⁴ At the onset of prophase, the diposomes, along with associated pericentriolar material, move to opposite sides of the nucleus and establish the bipolar mitotic spindle.²⁵

We recently have shown that the centrosomes of high-grade breast cancers do not follow this program of events.¹⁴ In breast tumor cells, centrosome duplication is uncoupled from the cell cycle, resulting in cells with numerous centrosomes, many of which are larger than normal. Tumor centrosomes typically show inappropriate levels of phosphorylated proteins, in contrast to normal centrosomes, which contain increased levels of phosphorylated proteins during mitosis.

Here we compare the ultrastructure of centrosomes of normal breast epithelial tissues and breast adenocarcinomas. These studies reveal dramatic abnormalities in the centrioles and centrosomes of breast tumor cells. These abnormalities include 1) supernumerary centrioles, 2) excess pericentriolar material, 3) disrupted cen-

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triolar barrel structure, 4) unincorporated microtubule complexes, 5) centrioles of unusual length, 6) centrioles functioning as ciliary basal bodies, and 7) mispositioned centrosomes. Structural centrosome abnormalities, most notably excess pericentriolar material, were associated with an increased frequency of abnormal mitoses as assessed by Ki-67-immunolabeled paraffin sections of the same tumors. The relevance of centrosome structure with regard to cell polarity, differentiation, bipolar and multipolar mitosis, and tumor progression is discussed.

Materials and Methods

Tissues

Tissues from 45 consecutive mastectomy and lumpectomy surgeries were collected according to an Institutional Review Board-approved protocol. Tissues were omitted from the analysis if patients had received previous chemotherapy or radiation therapy ($n = 6$), did not include primary invasive tumor ($n = 4$), were poorly preserved ($n = 3$), or were from male patients ($n = 1$). The remaining 31 tumors, which included two grade 2, nine grade 3, and twenty grade 4 specimens (Mayo histological grading scale), were analyzed. Six normal tissues from breast reduction surgeries were also analyzed.

Transmission Electron Microscopy Processing and Observation

Tissues were cut into small pieces and placed in fixative (4% formaldehyde, 1% glutaraldehyde in sodium phosphate buffer, pH 7.2) at 4°C for up to 36 hours. Tissues were further processed by postfixation in osmium tetroxide, *en bloc* staining with uranyl acetate, dehydration in ethanol, and embedding in epoxy resin. Thin sections were poststained with lead citrate and examined using a Philips CM10 Biotwin transmission electron microscope (Philips Electronic Instruments, Mahwah, NJ). Tissues were categorized according to centrosome location, number of centrioles in thin section, qualitative level of pericentriolar material, presence and arrangement of centriolar appendages, presence of primary cilia, variations on centriolar structure, and multipolar mitotic figures.

Light Microscopy and Mitotic Index Determination

Portions of tissues also were formalin-fixed and paraffin-embedded for light microscopy. Sections were immunostained using MIB-1 antibody against Ki-67 (Dako Corp., Carpinteria, CA). Ki-67 is a nuclear antigen that is present in late G1, S, G2, and mitotic cells, but is lacking in G0 and early G1 cells. Condensed chromosomes are stained intensely with this antibody, allowing for easy quantification of proliferative and mitotic cells and identification of abnormal mitotic figures. Proliferative index (PI) was calculated as the percentage of Ki67-positive

cells out of the total number of epithelial cells. A minimum of 200 cells was counted in defined fields of view using a 40× objective. Likewise, mitotic index (MI) was calculated as the percentage of mitotic cells in the same fields of view. When no mitotic cells were observed, the MI was calculated as <1 mitotic cell per the total number of cells observed. Because the frequency of abnormal mitotic figures is very low in most tissues, the abnormal mitotic index (AMI) was determined by scanning the entire section and counting the total number of mitotic cells and the total number of abnormal mitotic figures. The ratio of abnormal to total mitoses was then multiplied by the mitotic index to yield the AMI. These data are summarized in Figure 7. All tissues were scored blindly. Photographs were made using a Nikon FXA photomicroscope.

Centrin Immunofluorescence

A subset of tissues was selected for immunofluorescence studies. These tissues included one tumor with normal centrosome ultrastructure, one tumor with clusters of extra centrioles, two tumors with extra pericentriolar material, and two tumors with inverted polarity. Normal tissue used for immunofluorescence was from a different patient than that used in the ultrastructure studies. All tissues were frozen in liquid nitrogen within 30 minutes of surgical removal and stored at -70°C until use. Cryosections were immunostained with a monoclonal antibody against centrin, a centrosomal protein, as previously described.¹⁴ Sections were examined and photographed using a Nikon FXA epifluorescence microscope.

Results

Normal Breast Epithelium

Normal breast epithelial tissues were organized with a high cuboidal layer of luminal cells separated at intervals from the basement membrane by a discontinuous layer of myoepithelial cells (Figure 1, A and B). The nuclei of the luminal epithelial cells tended to be basal and the centrioles apical. Although apical, most often the position of the centrioles was eccentric; that is, they were located near the lateral junctional complexes of adjacent cells (Figure 1B). Although centrioles usually did not maintain an orthogonal orientation, they were typically close to each other (Figure 1, A and C). Occasionally, an extremely short primary cilium extended from the distal end of the mature centriole (Figure 1C). Fine striated rootlets infrequently were observed extending from the proximal ends of centrioles toward the base of the cell (Figure 1D). The striated rootlets were quite variable in extent and were not observed with most centrioles. Other than distal and subdistal appendages on the mature centriole and fine fibrillar material along the outer walls of the centriole barrels, little pericentriolar material was noted with the centrioles of normal luminal epithelial cells (Figure 1, A-D). Subdistal appendages were slightly more developed on the centrioles of the myoepithelial cells, and their primary cilia were longer than those of luminal epithelial

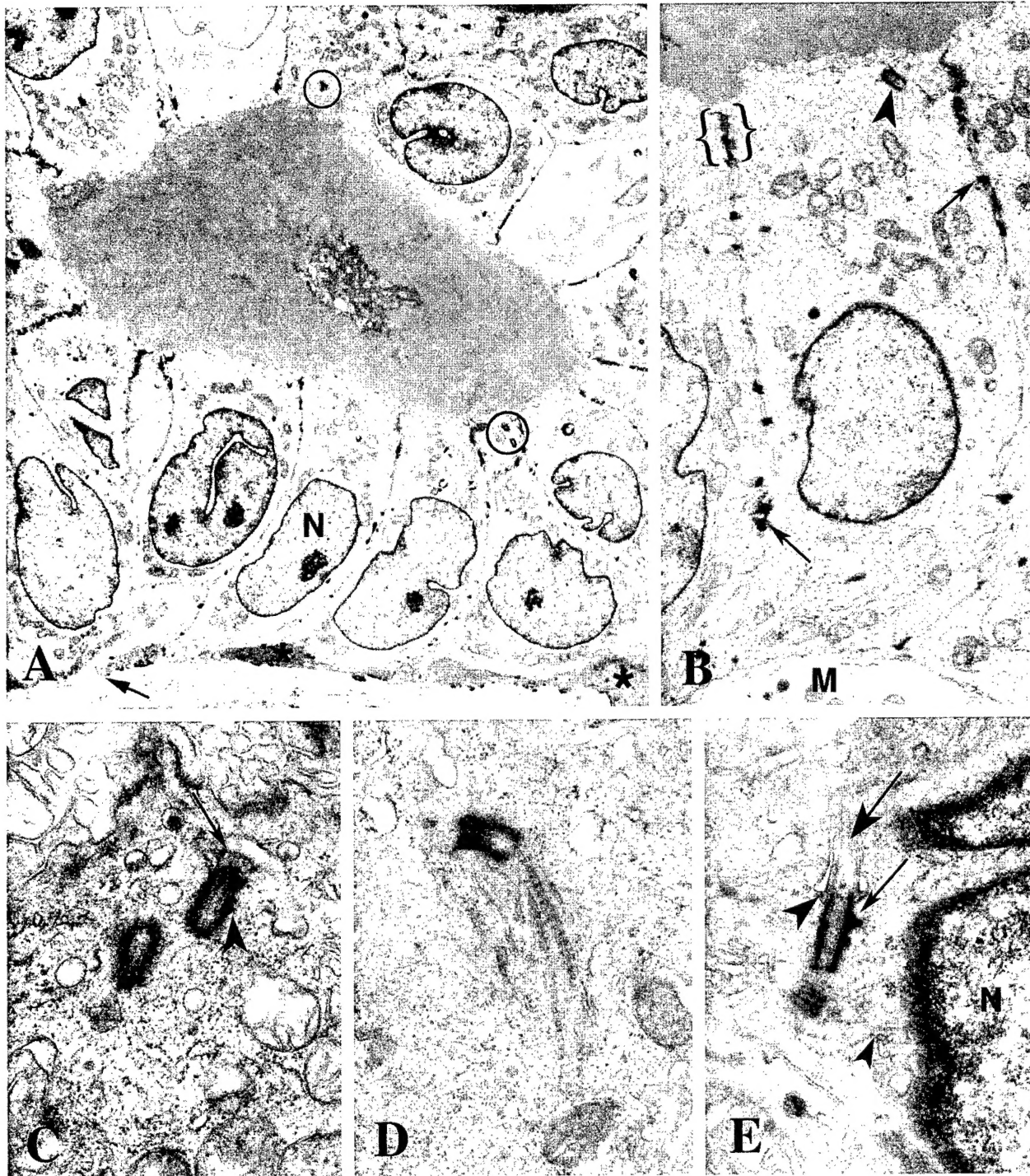


Figure 1. Normal breast epithelium. **A:** The normal breast ductal epithelium consists of a high cuboidal layer of luminal cells subtended by a discontinuous layer of myoepithelial cells (*) and basement membrane (arrow). The nuclei (N) are basal and the centrosomes (circled) are apical. **B:** Adjacent luminal epithelial cells are joined by lateral junctional complexes (brackets) near the apical membrane and desmosomes (arrows) between their lateral membranes. A single centriole (arrowhead) is located at the apex next to a junctional complex. A portion of a myoepithelial cell (M) is seen at the base of the luminal epithelial cell. **C:** The mature centriole of this nonorthogonal diplosome bears a short primary cilium (arrow) at its distal end in this luminal epithelial cell. A small subdistal appendage (arrowhead) is present on the mature centriole, whereas the immature centriole lacks appendages. Although very little pericentriolar material is present, the centrosomes do have a coating of fine fibers. **D:** A striated rootlet extends from the proximal end of this mature centriole toward the base of the luminal epithelial cell. **E:** Fine fibers (small arrowhead) extend between the diplosome and the nearby nucleus (N) in this myoepithelial cell. Distal appendages (large arrowhead) extend between the centriole and the plasma membrane at the site of primary cilium (large arrow) emergence. Subdistal appendages (small arrow) are prominent on the mature centriole. The immature centriole is seen in oblique section. Original magnifications, $\times 3500$ (A), $\times 8850$ (B), $\times 27,500$ (C), $\times 25,600$ (D), $\times 21,200$ (E).

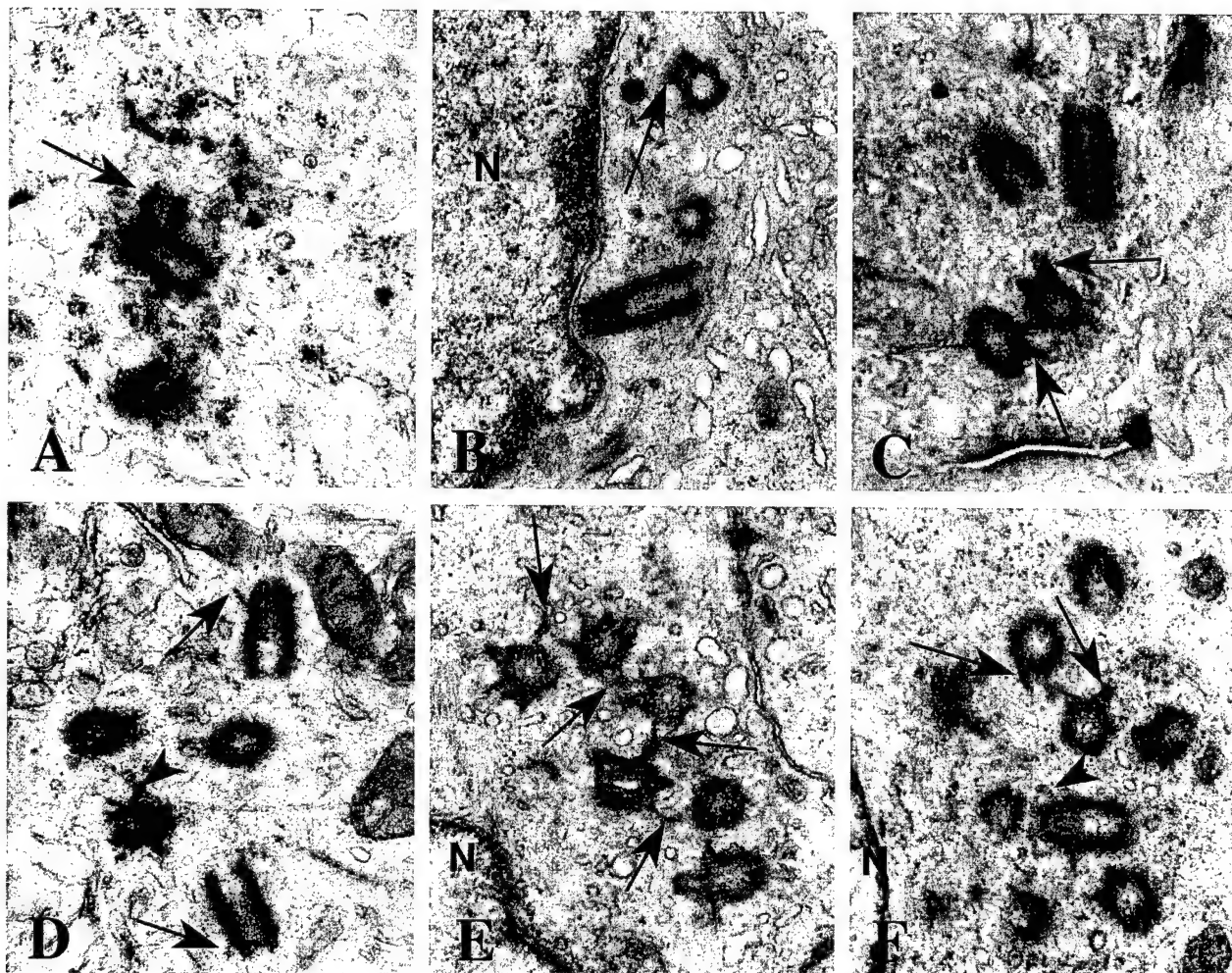


Figure 2. Supernumerary centrioles in breast tumors. **A:** A pro-centriole (arrow) is present at the proximal end of one of the two centrioles in this section. This pro-centriole is identifiable by its orthogonal orientation relative to the full length centriole and by the width of its lumen. Notice the electron opaque pericentriolar satellites surrounding the centrioles. **B:** Two centrioles are seen in cross section and a third is in longitudinal section. One centriole has subdistal appendages (arrow). All three are close to the nucleus (N). There is no orthogonal relationship between any of the three centrioles. **C:** At least two of these four centrioles have subdistal appendages (arrows). **D:** The barrels of these five centrioles are coated with a fine electron opaque material. Two centrioles have distal appendages (arrows) and at least one also has subdistal appendages (arrowhead). **E:** This group of six centrioles is linked by fine fibers between their subdistal appendages (arrows). The group is next to the nucleus (N). **F:** At least nine centriole profiles are present in this thin section. Subdistal (arrows) and distal (arrowhead) appendages are seen on many of the centrioles. The nucleus (N) is adjacent to this cluster of centrioles. Original magnifications, $\times 27,500$ (A and B), $\times 32,300$ (C and F), $\times 31,000$ (D), $\times 34,150$ (E).

cells (Figure 1, B and E). Unlike luminal epithelial cells, diplosomes of myoepithelial cells were located close to the nuclei. Filaments extended from the myoepithelial diplosome to the nucleus (Figure 1E); this was never observed in luminal epithelial cells. No centrosome abnormalities were observed in normal epithelial cells of the four reduction mammoplasties examined by electron microscopy.

Invasive Breast Tumors

Twenty-four of 31 invasive tumors contained centrosomes and that differed from those of normal breast cells in a variety of ways. Eleven tumors were characterized by centrosomes with more than two centrioles (Figures 2 and 3, A-C). In thin sections, these supernumerary centrioles ranged from a pair of centrioles with a single extra pro-centriole to a field of 9 centriole profiles (Figure 2, A-F). Often the extra centrioles were arranged in a group

and were closely linked by fine fibers extending between subdistal appendages (Figure 2, C, E, and F). Appendages normally associated with only the mature centriole were seen frequently with more than one centriole in these groups (Figure 2, C-F, and Figure 3A). Centrosomes with extra centrioles were most often located adjacent to the nucleus (Figure 2, B, E, and F), in contrast to normal luminal epithelial cells, in which the centrioles tended to be closer to the apical plasma membrane (Figure 1, A and B).

The amount of pericentriolar material and satellites associated with tumor centrosomes was variable, ranging from low levels similar to normal centrosomes (Figure 2, B-F), to moderate (Figure 2A) and excessive levels (Figure 3). In all, nine tumors had excess pericentriolar material, often in addition to extra centrioles. In some tumors this pericentriolar material had a distinct fibrogranular appearance (Figures 2A and 3) reminiscent of material associated with basal body formation in ciliated

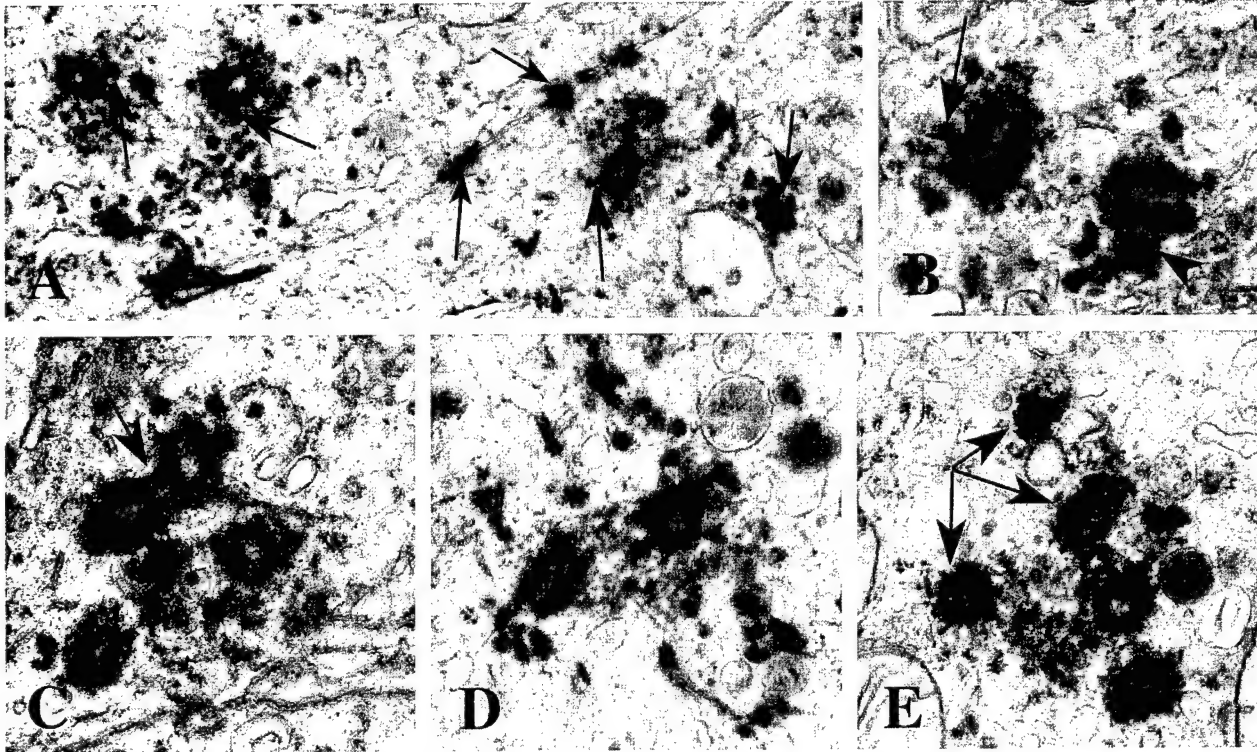


Figure 3. Excess pericentriolar material in breast tumors. **A:** Centrosomes in two adjacent cells are seen. Desmosomes (small arrows) tether the plasma membranes. All of the centriole profiles include subdistal appendages that are characteristic of mature centrioles (large arrows). Electron opaque fibrogranular material is present around both centrosomes. **B:** The barrels of these centrioles are coated with a dark granular material and pericentriolar satellites are present. One centriole has distal and subdistal appendages (arrow) while the other has a procentriole (arrowhead) associated with it. **C:** Fine electron opaque fibers coat the five centriole profiles seen in this section. Two orthogonal centrioles are connected by a dense parallel array of fibers (arrow). **D:** Two centrioles with numerous dark granules are present in this section. **E:** This centrosome contains one centriole and several masses (arrows) similar to generative complexes visible in this section. Original magnifications, $\times 17,900$ (A), $\times 31,650$ (B), $\times 28,000$ (C), $\times 28,700$ (D), $\times 27,650$ (E).

cells. Large granular masses, similar to generative complexes involved in ciliary basal body formation, were also observed in the pericentriolar material in some tumor cells (Figure 3E). Many centrioles were encased in electron opaque material pressed directly to the barrel of the centriole (Figure 3, B and C).

In addition to excessive pericentriolar material, two tumors had centrioles that were structurally defective in various aspects (Figure 4). Normal centrioles are composed of nine sets of triplet microtubules in which the A microtubule is complete and the B and C microtubules share protofilaments with A and B, respectively.²⁶ Unusual microtubule complexes were observed near complete centrioles in some tumors (Figure 4A). These microtubule complexes were not assembled into normal triplets nor arranged in a barrel shape; rather they were an assortment that included five or more microtubules with shared protofilaments embedded in amorphous electron-opaque material (Figure 4A). In one instance a centriolar microtubule triplet was displaced away from the centriole barrel, resulting in what has been termed an open ring centriole (Figure 4B). Unusually long centrioles (Figure 4D) were observed in one tumor. Primary cilia ranged from very short to well developed (Figure 4C).

Some tumors had regions of apocrine metaplasia in which luminal epithelial cells maintained normal apical/basal polarity, but had cytoplasmic beaks that projected into the lumen (Figure 5A). The beaks were bordered by

the apical plasma membrane that protruded well past the junctional complexes that mark the apical limit of the lateral plasma membrane. Beak cytoplasm contained numerous secretory vesicles, endoplasmic reticulum, and mitochondria. The centrosomes in these cells were near the junctional complexes and just apical to the nucleus, but not adjacent to the lumen as in normal luminal epithelial cells (Figure 5A). In one well differentiated grade 2 tumor with apocrine metaplasia, the beaked apocrine cells were mixed with ciliated cells. The ciliated cells also maintained apical/basal polarity, but along their apical membrane were numerous cilia with centrioles functioning as ciliary basal bodies (Figure 5B). These cilia and basal bodies were similar in location and appearance to those of normally ciliated cells such as ciliated respiratory epithelium. Microvilli also were located along the apical membranes of the ciliated cells (Figure 5B). The apical membranes of the ciliated cells did not protrude into the lumen as did the nonciliated beaked cells (Figure 5A). Both the ciliated and the beaked cells were in regions of tumors that were well differentiated.

Two tumors contained regions in which cells still maintained apical/basal polarity even in poorly differentiated and highly invasive tumors lacking a basement membrane (Figure 5C). The apical and lateral membranes were identified by their location relative to junctional complexes and the presence of microvilli on the apical membrane. In these instances, the cell apices often did not

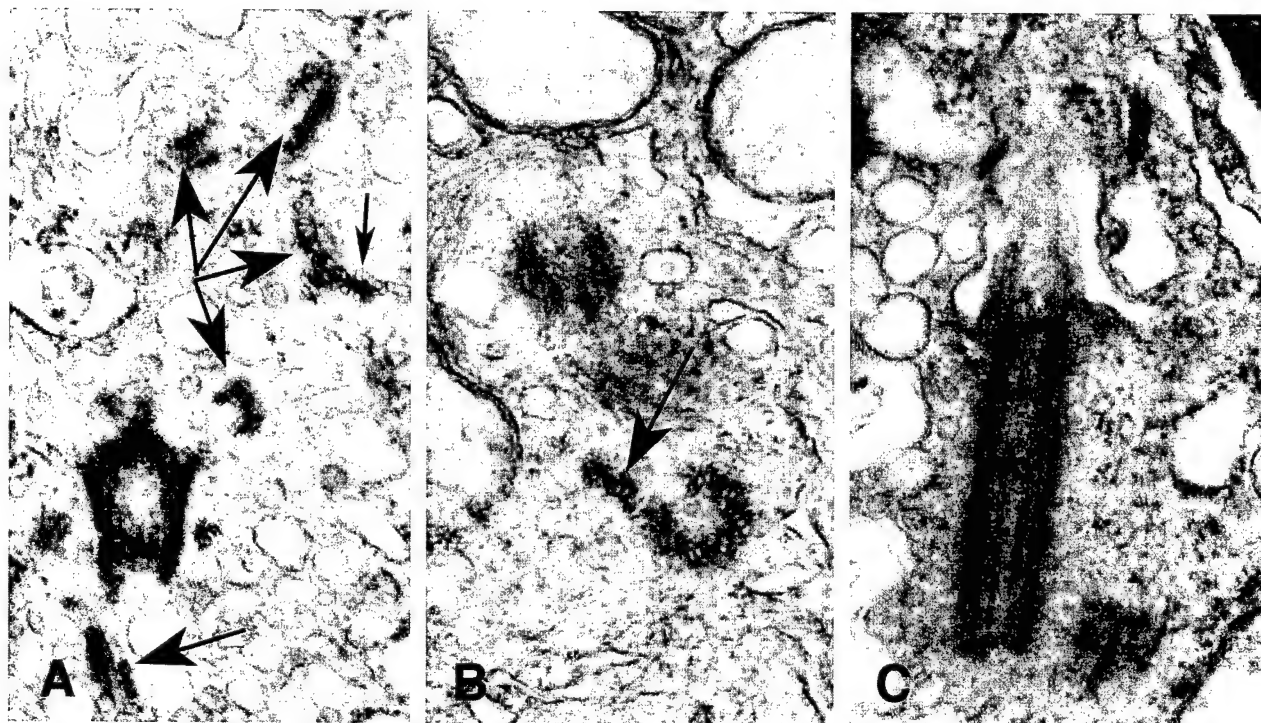


Figure 4. Abnormal centriole structure in breast tumors. **A:** Subdistal appendages are seen in this oblique section through a centriole. Numerous microtubule complexes (**large arrows**) are seen in various planes of section throughout the cytoplasm near the centriole. As is seen in cross section of the complexes, the individual microtubules share a portion of the wall of the neighbor microtubules (**small arrow**). **B:** The open-ring configuration of this centriole is shown in cross section. Two of the nine triplet microtubule complexes are splayed away from the centriole barrel (**arrow**). **C:** This centriole bearing a primary cilium (*) is nearly twice as long as normal centrioles. Original magnifications, $\times 54,500$ (**A**), $\times 59,625$ (**B**), $\times 47,700$ (**C**).

face a lumen, but instead faced collagen fibrils of the stromal connective tissue (Figure 5C). The centrosomes of these cells were normal in structure and were located next to the junctional complexes near the apical plasma membrane, but, because the apices face the stroma, the cell polarity was inverted.

Mitosis in Tumor Cells

Although mitotic figures were not observed in normal breast tissues, there were numerous mitotic figures present in four of the tumors examined transmission electron microscopy. Some mitotic figures appeared normal in thin section, having a typical metaphase plate and bipolar spindle (not shown), whereas others had significant abnormalities (Figure 6). A tripolar mitosis is shown in Figure 6A. Tracings of microtubules, spindle poles, and condensed chromosomes from six nonadjacent serial sections through the cell in Figure 6A are presented in Figure 6B. Analysis of the reconstruction in three dimensions revealed that one spindle pole was composed of two distinct but adjacent foci of microtubules, which perhaps resulted from their coalescence in prometaphase. Each spindle pole had at least two centrioles recognizable as distinct structures in these six nonadjacent thin sections. Many division figures were too bizarre for analysis in thin section.

Centrin Immunofluorescence

As previously described,¹⁴ normal breast tissues have an apically positioned pair of immunolabeled spots that correspond to the centrioles (Figure 6E). Pairs of spots also were observed in cells of the tumor with normal centrosome ultrastructure, although the tissue was anaplastic and centriole location appeared random (Figure 6F). Many cells in the tumor with numerous centrioles closely linked by fine fibers contained clusters of spots the size and shape of centrioles (Figure 6G), whereas spots of various sizes and shapes were present in cells of the tumors characterized by extra pericentriolar material (Figure 6H).

Proliferation and Mitotic Indices

Indices of proliferation, mitosis, and abnormal mitosis are summarized in Figure 7. Tissues were placed in one of four categories on the basis of tissue type and centriole/centrosome structure. Category I is comprised of all normal tissues from reduction mammoplasty. All six of these tissues had normal centrosome structure as assessed by immunofluorescence and/or electron microscopy. Category II consists of the nine tumors that have normal centriole/centrosome structure as assessed by immunofluorescence and electron microscopy. Category III contains twelve tumors with abnormal centriole/centrosome structure such as supernumerary centrioles or structur-

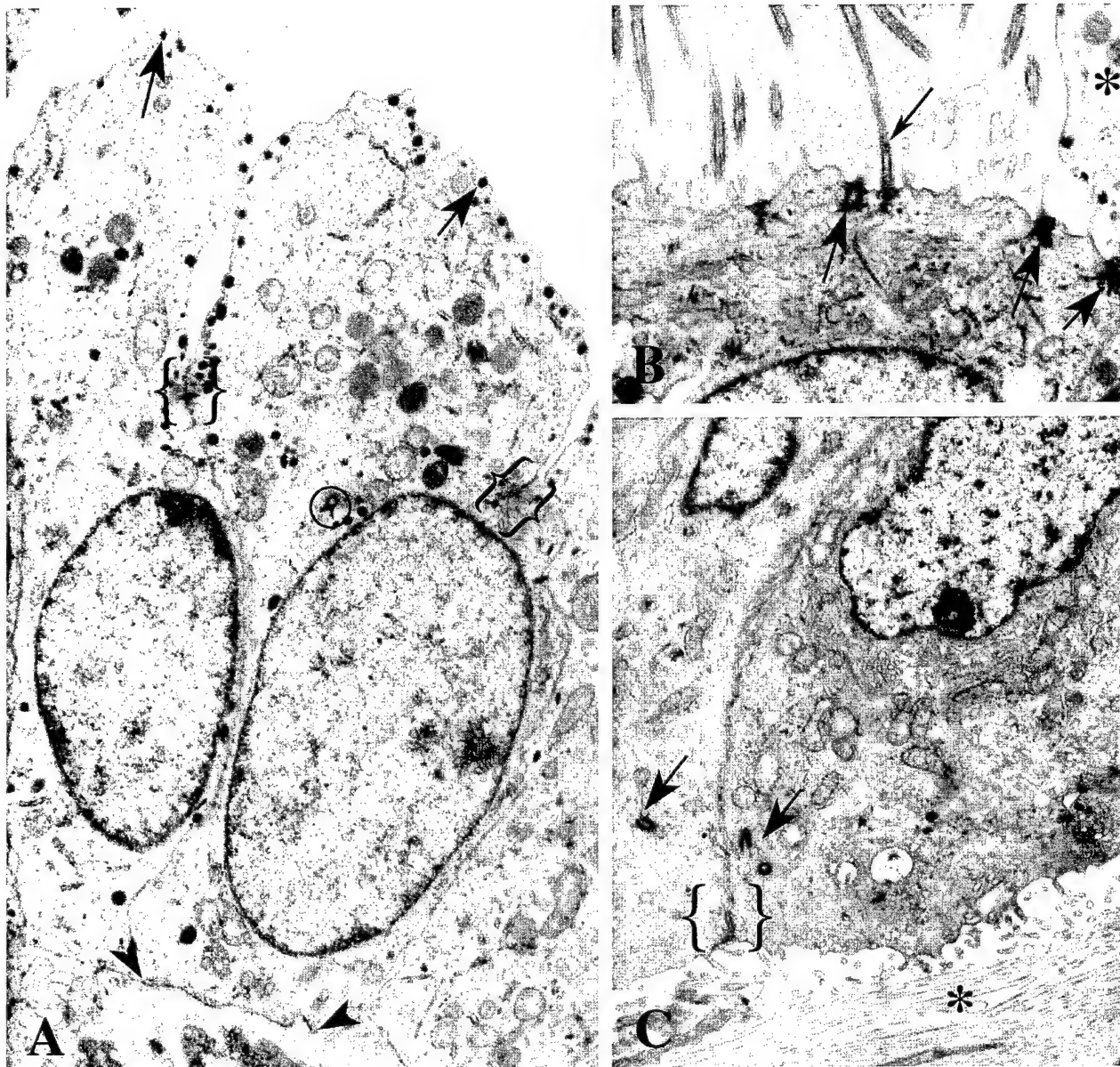


Figure 5. Positional centrosomal anomalies in breast tumors. **A:** Secretory granules (arrows) are present at the apical membrane of these cells displaying apocrine metaplasia. Junctional complexes (brackets) mark the transition from lateral to apical membrane domains. Apocrine beaks extend into the lumen of the duct. Notice the centriole (circled) near the apical end of the nucleus. These cells have apical/basal polarity and rest on a basement membrane (arrowheads). **B:** Extra centrosomes in this cell are inserted at the apical plasma membrane where they function as basal bodies (large arrows) for cilia (small arrow). Microvilli and cilia project into the lumen. The beak of an adjacent apocrine cell (*) is visible. The ciliated cell does not protrude into the lumen, as does the apocrine cell; but like its apocrine neighbor, it has apical/basal polarity and rests on a basement membrane (not visible in this figure). **C:** The two centrosomes (arrows) seen in adjacent cells are located near the junctional complex between these polarized cells (bracket). However the apical membrane domain with microvilli faces collagen (*) of the stromal tissue rather than the lumen of a duct. This invasive group of cells has ramified through the breast stroma and is not subtended by a basement membrane. The polarity of these cells is inverted, with the basal domains abutting the basal domains of other cells and the apical domains facing the stroma rather than a lumen. Original magnifications, $\times 8150$ (A), $\times 10,000$ (B), $\times 7900$ (C).

ally defective centrioles. Tumors with excess pericentriolar material in addition to centriole abnormalities are excluded from this category and placed in Category IV. Category IV contains seven tumors with excess pericentriolar material, regardless of other centriole/centrosome characteristics.

The six normal breast tissues (Category I, Figure 7) examined by light microscopy had a median PI of 5.3% as determined by Ki67 immunostaining. These normal tissues had a median MI of 0.00% (mean mitotic index =

0.03%) based on the total of 4238 epithelial cells observed. On examination of entire histological sections from all six tissues, only two contained identifiable mitotic figures, and no abnormal mitotic figures were observed. Of the nine tumors with normal centriole/centrosome ultrastructure (Category II, Figure 7), five contained no abnormal mitotic figures and four did, yielding a median AMI of 0.00% (mean = 0.16%). The median PI, MI, and AMI of Category II tumors were not significantly different from Category III tumors.

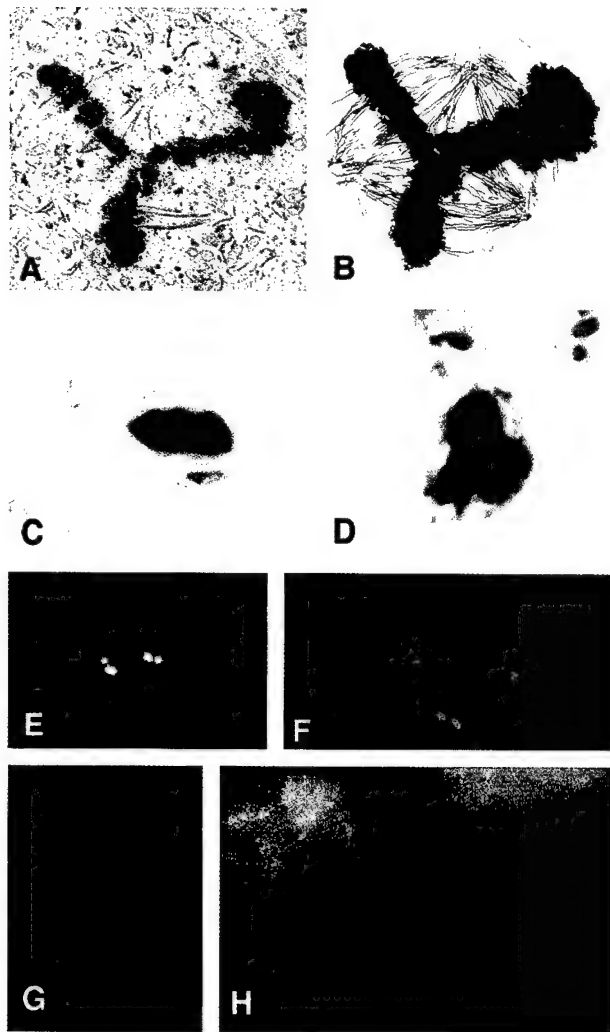


Figure 6. Multipolar mitoses and centrin immunofluorescence. **A:** This section through a symmetrical tripolar mitotic cell shows part of the metaphase plate and portions of the tripolar spindle. **B:** Tracings of microtubules (red), spindle poles (green), and condensed chromosomes (blue) from six nonadjacent serial sections through the cell shown in **A** are shown in this overlay. The upper spindle pole appears to contain two separate, but adjacent, microtubule foci that have coalesced. **C:** A normal metaphase plate is shown in this Ki-67 immunostained paraffin section of a breast tumor. **D:** A tripolar metaphase cell immunolabeled with Ki-67 is shown in this tumor section. **E:** In normal breast epithelium, the centrosomes appear as distinct pairs of spots when labeled with antibodies against centrin. Centrosomes of two adjacent cells are shown in this cryosection. **F:** In this tumor characterized with normal centrosome ultrastructure, the centrosomes are similar to those of normal tissue when immunolabeled using antibodies against centrin. **G:** Centrin immunofluorescence of the same tumor shown in Figure 2, E and F, reveals a cluster of centriole-sized spots as well as a normal looking pair of spots. By transmission electron microscopy this tumor had up to 9 centrosomes in a single thin section, but no excess pericentriolar material. **H:** Centrin immunofluorescence of the same tumor as shown in Figures 3D and 6A reveals numerous large, amorphous spots. By transmission electron microscopy, centrosomes of this tumor contain excess pericentriolar material and extra centrosomes. Original magnifications, $\times 160$ (**A** and **B**), $\times 925$ (**C** and **D**), $\times 2050$ (**E-H**).

The Category IV tumors, characterized by the presence of excess pericentriolar material, had the highest median frequencies of proliferation, mitosis, and abnormal mitosis (28.2%, 0.71%, and 0.46%, respectively). Category IV values, with the exception of the PI relative to Category III, were significantly different from the values of all other categories.

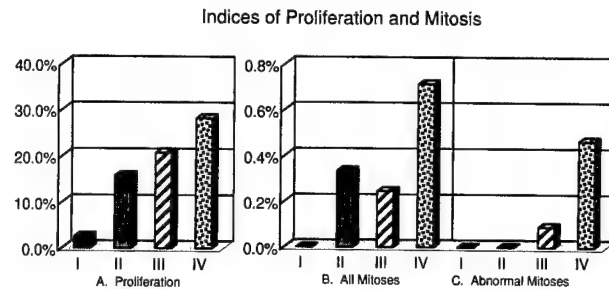


Figure 7. Indices of proliferation and mitosis. Tissues were placed in categories as follows: I (solid black bars), six normal tissues from reduction mammoplasties with normal centriole/centrosome structure (all normal tissues examined fell in this category); II (solid gray bars), nine tumor tissues with normal centrioles/centrosomes; III (striped bars), twelve tumor tissues with abnormal centrioles (this includes tissues with supernumerary centrioles and those with centriole defects, but excludes those with excess pericentriolar material); and IV (stippled bars), seven tumor tissues with excess pericentriolar material, regardless of centriole defects. The Wilcoxon Rank Sum Test was used to determine statistical significance. Median values are plotted. **A:** The proliferation index of category I (normal tissue) is significantly lower than the other three categories and that of category IV (tumors with excess pericentriolar material) is significantly greater than categories I and II, but not III. Categories II and III are not significantly different from each other. **B** and **C:** Category IV has significantly higher frequencies of mitosis and abnormal mitosis than the other three categories. The other categories were not significantly different from each other.

Discussion

The centrosome functions to nucleate and organize microtubules; during interphase the centrosome is the primary microtubule organizing center, and during mitosis duplicated centrosomes serve as mitotic spindle poles.¹⁹ We found that centrosomes in normal breast tissue are apical and usually adjacent to the junctional complex, whereas nuclei are basal. Very little pericentriolar material is associated with these centrosomes. As is seen in other polarized epithelial cells,²⁷ centrioles may separate a short distance from each other after losing their orthogonal orientation, and the mature centriole may form a short primary cilium. In addition, centrioles occasionally bear a striated rootlet.

Only by selecting breast biopsy tissue from premenopausal women in the luteal phase of the menstrual cycle was Ferguson²⁸ able to investigate mitosis in normal breast parenchyma. In these normal cells, very little pericentriolar material was associated with the spindle poles. The normal tissues in the present study were not selected according to the phase of menstrual cycle, and no mitoses were observed by transmission electron microscopy or by light microscopy. However, normal breast epithelium does maintain a population of proliferating cells that immunostain with antibodies to Ki67; our median PI value of 5.3% in normal breast epithelium is within the range of published values.²⁹ In agreement with our observations on interphase cells by immunofluorescence and by transmission electron microscopy, Ferguson²⁸ noted that centrioles of normal interphase cells were apical and not associated with the basal nuclei. Likewise, primary cilia have previously been noted in myoepithelial cells.³⁰

Centrosomes undergo changes throughout the cell cycle.²¹⁻²⁴ The nuclear and centrosome cycles are synchronized by checkpoints that prevent DNA reduplication before karyokinesis and prevent centrosome reduplication.

tion before anaphase. In certain normal cell types such as binuclear mouse hepatocytes³¹ and human megakaryocytes,³² synchrony between the nuclear and centrosome cycles is maintained even in the absence of cytokinesis, resulting in polyploid cells with centrosome numbers appropriate for the level of ploidy. Due to the numerous centrosomes arranged around the polyploid nucleus, megakaryocytes lack apical/basal polarity, although they do have a radial organization. In contrast, cancer cells have asynchronous nuclear and centrosome cycles, often resulting in multicentrosomal aneuploid cells that lack apical/basal polarity and appear disorganized.

We have shown that centrosomes and centrioles of most human breast tumors (24 of 31 analyzed) display a range of significant structural and functional abnormalities. Breast tissues can be divided into four categories: normal tissue with structurally normal centriole/centrosomes (Category I), tumors with structurally normal centriole/centrosomes (Category II), tumors with centriole-based abnormalities (Category III), and tumors with excess pericentriolar material (Category IV). Category IV tumors are associated with significantly increased frequencies of both normal and abnormal mitoses. Cells having no visible centrosome abnormality are also present in all tumors. Some abnormalities may be related to loss of synchrony between the centrosome cycle and nuclear cycle.

Tumor cells that become ciliated retain apical/basal polarity and tend to be well differentiated. These tumors are included in Category III. Ciliated cells have been described infrequently in breast carcinomas.³³ These multiple centrioles probably arise through the same acentriolar basal body neogenesis that occurs in normal ciliated epithelial cells.³⁴⁻³⁷ In effect, these cells differentiate into the wrong cell type, resulting in metaplasia rather than anaplasia. These ciliated breast tumors have PI and MI of 20% and 0.2%, respectively, similar to normal breast epithelium. The ciliated cells, like normal ciliated epithelial cells, probably are terminally differentiated and remain in G0 of the cell cycle. Therefore, the production of centrioles that function as ciliary basal bodies may be a relatively harmless structural alteration with no adverse implications for genetic stability.

Open-ring centrioles and centrioles missing triplet microtubules (MTs) occur in some Category III tumors. Although these structures are similar to those present during basal body formation in hamster ciliogenesis,³⁸ no cilia are present in these tumors. Disrupted centriole barrels similar to open-ring centrioles have also been observed as a consequence of infection with and treatment with DNA-binding dyes,³⁹ and DNA-binding dyes have been shown to induce multipolar mitoses in cultured cells.³⁹ However, in the present study, open ring centrioles are not associated with an increase in the frequency of multipolar mitoses.

Unusual microtubule complexes embedded in dark amorphous material were also noted in one Category III tumor. The PI, MI, and AMI of this tumor are not significantly different from those of tumors with normal centrosome structure. These novel structures have not been

described previously, and their importance is not understood. They may be a further indication that the mechanics, as well as timing, of centriole formation is not well regulated in tumors.

Some tumors (11 of 31 studied) produce extra centrioles that do not serve as ciliary basal bodies. In some cells of these Category III tumors, centrioles often appear linked closely together by fine fibers and remain near the nucleus. These tumors are anaplastic; ie, they are not as differentiated as tumors that produce cilia and do not retain apical/basal cell polarity. The presence of procentrioles along the proximal walls of mature centrioles indicates that these extra centrioles arose through template driven duplication rather than through acentriolar neogenesis typical of basal body production in ciliated cells.³⁵ Fine fibers linking the centrioles in tumors are similar to those described linking the pair of centrioles of a diplosome,⁴⁰ further supporting the idea that they originate as procentrioles associated with a mature centriole. Because template-driven centriole duplication normally occurs only once per nuclear cycle, these cells have lost the synchrony between the nuclear cycle and the centrosome cycle. As long as the centrioles remain linked together, they may function as one large centrosome in an interphase cell. However, if these large centrosomes separate into more than two spindle poles at the onset of mitosis, it is likely that chromosomal missegregation will occur, resulting in aneuploidy. Indeed, the frequency of abnormal mitoses is quite variable among these tumors, indicating that most cells with extra centrioles are capable of forming bipolar spindles.

Other tumors (9 of 31 studied, 7 of which were available for proliferation and mitotic index determination) accumulate excess pericentriolar material with their centrosomes and variable numbers of extra centrioles (Category IV tumors). The nature of the pericentriolar material is reminiscent of fibrogranular material and generative complexes associated with acentriolar as well as centriolar basal body formation.^{34-37,41} However, no cilia are observed and the randomly positioned centrioles are not located near the plasma membrane. This accumulation of excess pericentriolar material may be the result of overexpression of centrosomal proteins or the reorganization of material that is normally dispersed within the cytoplasm.^{14,42,43} Increased levels of γ -tubulin,^{14,17} pericentrin,¹⁵ and centrin¹⁴ have been demonstrated in abnormal centrosomes in human tumors, and it is likely that other centrosomal proteins are present in increased levels as well. γ -tubulin-containing complexes located in the pericentriolar material are the site of microtubule nucleation, and as such are key to centrosome function.⁴⁴ We have shown that tumors with excess pericentriolar material are highly anaplastic and have lost cell polarity. These Category IV tumors tend to have higher median frequency of abnormal mitoses (0.46%) compared to tumors with other centrosome abnormalities (0.09%). This higher frequency of abnormal mitoses in tumors with extra pericentriolar material suggests that the regulation of accumulation of centrosomal proteins is more critical than regulation of centriole duplication for proper centrosome function during the cell cycle.

Some cells have more than two centrosomes that can function as spindle poles, yielding atypical multipolar mitoses. Atypical mitoses have been observed in breast tumors and other pathological specimens such as ulcerative colitis⁷ and a mouse model of pancreatic cancer.³ Multipolar mitoses were observed in several breast tumors in the present study. Aberrant mitoses such as these may arrest in metaphase, with the cells eventually undergoing apoptosis. In some instances, however, a selective advantage may be conferred to one of the daughter cells, leading to a clone of cells with chromosome gains and/or losses.

Serial sectioning through mitotic tumor cells showed that spindle poles are sometimes composed of more than one focus of microtubules. These spindle poles likely resulted from the coalescence of two or more centrosomes before metaphase. Coalescence of centrosomes could allow the formation of a bipolar spindle in a cell having extra centrosomes. Coalescence of extra centrosomes may be a mechanism by which cells can minimize the rate at which aneuploidy develops in tumors. Because compounded aneuploidy ultimately would be a self-limiting characteristic of tumors, a proportion of bipolar mitoses must be maintained for tumor growth.

The centrosomal abnormalities described here in breast tumor cells reflect changes in the status of cell and tissue differentiation of the tumors. Differentiated tumors have centrosomes of more normal appearance that are either mislocated, as in the tumors with inverted cell polarity, or perform a normal function not typical of mammary epithelial cells, such as producing ciliary basal bodies in tumors displaying apocrine metaplasia. Centrosome abnormalities are characteristic of poorly differentiated anaplastic tumors that have lost checkpoint synchronization of nuclear and centrosome cycles. This loss is reflected in centrosome defects and multipolar mitoses. As recognized by Boveri² earlier in this century, defective centrosomes may decrease the fidelity of chromosome segregation during multipolar mitoses. Consequently, centrosome abnormalities such as those described here may confer a mutator phenotype to tumor cells. As is the case for the molecular mutator phenotype, most mutated progeny will not be viable, but occasionally progeny with a selective advantage will emerge and thrive, and thus the tumor progresses to a more aggressive state.

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The Role of the Centrosome in the Development of Malignant Tumors

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I. Introduction

The fundamental characteristic of the centrosome is that it embodies the major microtubule organizing center (MTOC) of the cell. As such, the centrosome determines the number and polarity of cytoplasmic microtubules. Once in each cell cycle, the centrosome is duplicated to give rise to two centrosomes (i.e., the mitotic spindle poles) that organize the microtubule array of the mitotic spindle and thereby make possible equal segregation of sister chromatids into each of two daughter cells at the time of cell division. The centrosome also plays a role in organizing cytoplasmic structure in interphase cells through its influence on the spatial array of microtubules. Recent observations have implicated defects in centrosome behavior in the progression of malignant tumors. In particular, centrosome defects may be the causative basis of *aneuploidy* (inappropriate number and combination of chromosomes) and *anaplasia* (loss of tissue organization and architecture). New insights into centrosome composition, structure,

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assembly, and regulation of centrosome duplication are bringing full circle early theories on the role of centrosome defects in the origin of malignant tumors (Andersen, 1999; Marshall and Rosenbaum, 1999; Karsenti, 1999; Brinkley and Goepfert, 1998; and elsewhere in this volume). Thus, a clearer understanding of centrosomes and cancer is beginning to emerge. This review concentrates specifically on centrosome defects seen in malignant tumors and the role that they may play in tumor progression.

A. Recognition of Cancer as a Disease of Cells and the Influence of Centrosomes in Tumor Progression

The earliest recorded historical mention of the problem of cancer is seen in the Edwin Smith Surgical Papyrus dating to the 17th century B.C. in Egypt (Breasted, 1930). In this ancient record a tumor (*ben.wet*) of the breast is described as a "swelling spread over the breast . . . (which is) without fever, with no granulation or fluid . . . having a touch or surface like that of a kind of ball, . . . likened to an unripe fruit which is hard and cool to the touch." Four millennia later in 1838, J. Müller found tumors to be "composed of cells, each containing nuclei and nucleoli" (as cited in Ewing, 1919). In his classic treatise on cellular pathology, Rudolph Virchow established the doctrine of *Omnis cellula e cellula* and recognized the cell as the basic element of pathological processes. Yet, Virchow, contrary to his own declaration on the origin of cells, believed that cancer originated from a fluid blastema of connective tissue (Virchow, 1860). It was not until early in this century that the cellular basis of cancer was generally accepted. Theodor Boveri (1914) wrote that "the problem of tumors is a cell problem" and that "every theory of malignant tumors is wrong which does not take into account its unicellular origins." Boveri further recognized that in order for normal development to proceed, cells must have a complete set of chromosomes. Based on his studies on the similarity between abnormal sea urchin embryo development following dispermic fertilization and cell anomalies seen in cancer, Boveri proposed the hypothesis that malignant tumors arise through centrosome defects that result in improper cell divisions and give rise to aneuploidy (1914). Galeotti also reported early in this century that asymmetric mitosis in tumors may result from "secondary subdivision of centrosomes, one of which may divide into as many as four parts, each forming attraction spheres. Under these conditions the migration of chromosomes is often delayed or unequal" (as cited in Ewing, 1919, page 39). Thus, early in the 20th century, the two defining properties of malignant tumors (alteration in tissue architecture and genetic instability) were established as hallmarks of cancer and their anatomic and causative roots were suspected to lie in inappropriate centrosome behavior. More

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recently, progress in understanding the molecular basis for tumor progression has focused on the role of dominant acting oncogenes, the loss of action of tumor suppressor genes, and their control of critical cell cycle events.

B. Tumor Biology Primer

A tumor is an autonomous new growth of tissue (Ewing, 1919). Beyond this seemingly simple definition, it is difficult to add characteristics of tumors that apply to all cancers. This is largely because human tumors vary greatly in their properties. *Neoplasms* (new growths) can arise in any tissue, and they differ from normal tissue regeneration and repair in that they are abnormal growths that persist and continue to grow after cessation of the stimuli that evoked their initial appearance (Lieberman and Lebvitz, 1996). Neoplasms may be *benign*, slow-growing, and relatively innocuous in that they remain at their site of origin. Benign tumors are generally encapsulated, well differentiated, and show normal nuclear structure. Nevertheless, if they secrete hormones or other factors affecting distant targets, hemorrhage, or otherwise compromise vital functions through location and sheer mass, "benign" tumors may have deleterious and even lethal consequences. *Malignant* tumors, on the other hand, are distinct from benign tumors in that malignant tumor cells migrate away from their original site of growth, initially through *invasion* of surrounding tissue, and then through *metastasis* to distant sites where they establish new tumors (Nowell, 1976; Loeb, 1991). The degree to which tumor cells retain differentiated characteristics of their tissue of origin is called *tumor grade*. Thus, tumor grade is a histopathological term and its designation is somewhat subjective depending on the training of the observer. A low-grade tumor is well differentiated, whereas a high-grade tumor tends to be *anaplastic*. Nuclear grade, another histopathological term, refers to the degree to which nuclear shape, staining, and location of heterochromatic DNA resembles that of normal tissue. *Tumor staging*, on the other hand, is a prognostic tool that is based on the size of the primary tumor, lymph node involvement, and the presence or absence of metastasis to distant sites.

Transformation is the term used to describe the conversion of normal cells to those with abnormalities in cellular appearance and growth regulation in tissue culture (*morphological transformation*) seen for cancer cells. This typically includes acquisition of unlimited growth potential, alteration in cell morphology, loss of contact inhibition of growth, growth in the soft agar colony assay, and loss of dependence on growth factors or serum, among others (Roberts, 1989). *Malignant transformation* has the further requirement that the cells can produce a tumor in an appropriate animal model. A confounding feature of tumors is their heterogeneity; tumors

consist of different populations of cells with diverse characteristics (Fidler and Kripke, 1977). Although most tumors are monoclonal in origin, subpopulations of cells arise that differ in immunogenicity, growth rate, karyotype, receptor status, susceptibility to cytotoxic drugs, and metastatic potential. For the genesis of aggressive malignant tumors, continual selection operates to drive a cascade of (sequential) steps that involve multiple tumor-host interactions. These changes are often irreversible and typically require multiple genetic lesions in key oncogenes and tumor suppressor genes.

Because the nominal rate for genetic mutation in somatic cells appears to be insufficient to generate the number of genetic changes found in most cancers, a "mutator" phenotype has been proposed that acts to specifically increase the level of genomic instability during tumor progression (Loeb, 1991; Nowell, 1976). Recent experimental studies using chemically transformed Chinese hamster embryo cells and analysis of karyotypic instability in human colon cancer cell lines has demonstrated that the degree of genetic instability is proportional to the degree of aneuploidy (Duesberg *et al.*, 1998; Lengauer *et al.*, 1998; Li *et al.*, 1997). Aneuploidy alone may be sufficient to explain genetic instability and the resulting karyotypic and phenotypic heterogeneity seen in cancer cells. Genomic instability, that is, the alteration in chromosome number through loss or gain of whole chromosomes (*aneuploidy*), chromosome translocation, gene amplification, and mutation, is a characteristic of solid tumors (Cheng and Loeb, 1997). Although certain genetic alterations may be common for particular tumor types, multiple genetic alterations are required for the full development of the cancer phenotype, and in some tumors these changes may follow a progressive pattern of acquisition (Vogelstein *et al.*, 1988). While the most frequent outcome of aneuploidy is cell death, some aneuploid cells may gain selective growth advantage, and it is the descendants of these cells that go on to develop the tumor.

II. Centrosome Defects and Abnormal Mitoses in Cancer

Abnormal mitotic figures, including multipolar and monopolar mitoses and lagging chromosomes, are easily discerned in standard histological slides used for diagnosis, and their presence is often noted in tumors (Koss, 1992; Pritchard and Youngberg, 1993; Haferkamp *et al.*, 1999; Remstein *et al.*, 1999; Tomaszewski *et al.*, 1999; Zamecnik and Michal, 1999). Although not always recognized as such, multipolar and monopolar mitoses are a direct consequence of centrosomal defects. Respectively, multipolar and monopolar mitoses result from extra centrosome duplication and lack of centrosome duplication or separation prior to mitosis. Cells in which some components of cell cycle checkpoint control remain intact may arrest in mitosis and

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eventually undergo apoptosis or necrotic cell death in response to abnormal spindle formation. However, daughter cells that do result from such mitoses most often are aneuploid or polyploid, and it is likely that many are not viable. Among those aneuploid and polyploid cells that do survive exists the potential for enhanced growth that could lead to tumor progression. Thus, abnormal centrosome number and function may confer to cells a "mutator" phenotype as first described by Nowell (1976).

III. Amplified Centrosomes and Aneuploidy

The relationship between an abnormal number of centrosomes and development of aneuploidy has been demonstrated in a mouse model of pancreatic cancer induced by simian virus 40 tumor antigen (Levine *et al.*, 1991). This tumor model is characterized by the sequential appearance of tetraploid and then aneuploid cell populations. When examined by transmission electron microscopy, interphase tetraploid cells contained 5-11 centrioles, and 5 or more centrioles were observed in mitotic cells, at least 1 of which was tripolar. The authors speculated that extra centrioles predisposed cells to form multipolar mitotic spindles, yielding aneuploid daughter cells associated with subsequent tumor development. A similar phenomenon was observed in cultured cells transfected to express the *Vpr* gene of human immunodeficiency virus 1 (HIV-1) in the presence of tetracycline. *Vpr* expression induced multipolar mitoses and aneuploidy (Minemoto *et al.*, 1999). The effects of this viral gene on centrosome structure and function, leading to aneuploidy through the formation of multipolar mitoses, may be a mechanism for the cancer predisposition associated with HIV-1 infections.

The presence of extra centrosomes or MTOCs prior to mitosis does not necessarily commit the cell to multipolar mitosis. For example, cultured N115 mouse neuroblastoma cells contain many separate MTOCs during interphase (Sharp *et al.*, 1982; Ring *et al.*, 1982). During prometaphase, the MTOCs gather into clusters and chains, which by metaphase assemble into two groups, one group residing at each pole of the bipolar spindle (Ring *et al.*, 1982). Serial reconstruction of spindle poles in mitotic cells of human breast tumors has revealed a similar clustering of centrosomes (Lingle and Salisbury, 1999). However, because in one instance a cell retained three spindle poles in spite of centrosome clustering, it must be assumed that the clustering process is not 100% efficient in forming bipolar spindles when multiple centrosomes or MTOCs are present (Fig. 1). Never the less, clustering of extra centrosomes at the spindle poles during mitosis can minimize the possibility of aneuploidy in cells containing amplified centrosomes.



Figure 1 Reconstruction of multipolar mitosis. This reconstruction is from six transmission electron micrographs of nonadjacent serial sections. The condensed chromosomes (blue) are arranged in a triskelion with three spindle poles (green) generating three sets of spindle microtubules (red). The spindle pole near the 12 o'clock position is actually a cluster of two groups of centrioles generating two slightly separate microtubule foci that function as one spindle pole. Even though some of the supernumerary centrioles were clustered at one spindle pole, this cell has three spindle poles. Daughter cells from mitoses such as these will be aneuploid. (From Lingle and Salisbury, *American Journal of Pathology*, in press.)

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As shown by immunofluorescence and immunohistochemical studies, centrosome amplification is a feature common to many human tumors such as breast tumors (Lingle *et al.*, 1998; Pihan *et al.*, 1998; Carroll *et al.*, 1999), astrocytoma, lung tumors (Pihan *et al.*, 1998), neuroectodermal tumors (Weber *et al.*, 1998), squamous cell carcinomas of the head and neck (Carroll *et al.*, 1999), and pancreatic tumors (Sato *et al.*, 1999) (Fig. 2). These studies have utilized numerous marker antibodies to detect amplified centrosomes, including human autoimmune sera and antibodies against centrin, pericentrin, γ -tubulin, and MPM-2 epitope, indicating that numerous centrosomal proteins are concomitantly overexpressed in tumors. In human breast tumors, not only are tumor centrosomes amplified, they contain levels of phosphorylated centrosomal proteins that are inappropriately high for interphase cells (Lingle *et al.*, 1998). *In vitro* experiments have demonstrated that amplified tumor centrosomes are functional MTOCs (Lingle *et al.*,

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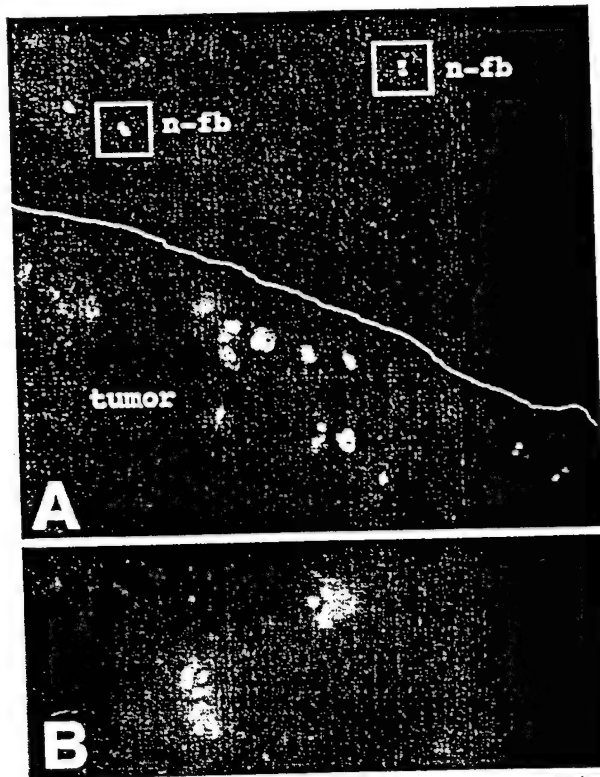


Figure 2 Immunofluorescence staining of centrosomes in human breast tumors. (A) A region of an adenocarcinoma of the breast stained for centrin showing excessive staining of centrosomes in the area of a tumor (below the marked line) and normal staining of pairs of centrioles in fibroblasts of the surrounding connective tissue. (B) Supernumerary centrioles in two adjacent cells of another tumor, stained for centrin. Each cell has eight or more centrioles.

1998; Pihan *et al.*, 1998), and breast tumor centrosomes exhibit a significantly greater than normal capacity to nucleate microtubules even at times when the tumor cells are not in mitosis (Lingle *et al.*, 1998). These facts indicate that in addition to the deregulation of centrosome duplication in tumor cells, the function and activity of centrosomes is not synchronized with the cell cycle.

IV. Excess Pericentriolar Material Is Associated with High Frequency of Abnormal Mitoses

In a recent electron microscopic comparison between centrosomes of normal human breast tissue and invasive breast tumor centrosomes, it was

noted that tumors frequently displayed ultrastructurally abnormal centrosomes, whereas centrosome abnormalities were rarely observed in normal tissues (Lingle and Salisbury, 1999). Tumor-associated centrosome abnormalities included (1) supernumerary centrioles; (2) excess pericentriolar material; (3) disrupted centriole barrel structure; (4) unincorporated microtubule complexes; (5) centrioles of unusual length; (6) centrioles functioning as ciliary basal bodies; and (7) mispositioned centrosomes. These alterations are associated with changes in cell polarity, and changes in cell and tissue differentiation, in addition to chromosome missegregation through multipolar mitoses. Significantly, of these seven centrosome abnormalities, the presence of excess pericentriolar material had the strongest association with a high frequency of abnormal mitoses. Usually, but not always, supernumerary centrioles were present along with excess pericentriolar material (Fig. 3). However, the presence of supernumerary centrioles in the absence of excess pericentriolar material did not significantly correlate with higher frequencies of abnormal mitoses. This means that unregulated centriole duplication by itself is likely insufficient to create multipolar mitoses; pericentriolar proteins must accumulate in excess at the centrosome to support the formation of multipolar mitotic spindles.

V. Centrosome-Associated Kinases and Cancer

Centrosome-associated kinases, including members of the aurora kinase family and the Polo-like kinases (PLK), are likely candidates for increased activity and/or accumulation at tumor centrosomes. These kinases are located at the centrosome or spindle pole in cell-cycle-dependent manner. As discussed in more detail elsewhere in this volume, these kinases are involved in regulating centrosome function and duplication, and their overexpression is associated with the development of aneuploidy (Sen *et al.*, 1997; Bischoff *et al.*, 1998; Zhou *et al.*, 1998). It has been suggested that aurora2 (a.k.a. BTAK/STK15), along with aurora1 and PLK1, may form a centrosome-associated kinase cascade whose disruption leads to genomic instability and chromosome defects (Bischoff *et al.*, 1998). BTAK/STK15 is overexpressed in breast tumors (Tanaka *et al.*, 1999; Zhou *et al.*, 1998), colon tumors (Bischoff *et al.*, 1998; Katayama *et al.*, 1999), and numerous tumor-derived cell lines (Zhou *et al.*, 1998). Overexpression of BTAK/STK15 in NIH 3T3 cells induces centrosome amplification and cell transformation (Zhou *et al.*, 1998). Most importantly, overexpression of STK15/BTAK in MCF10A cells (which are near diploid and have a normal mitotic apparatus) results in centrosome amplification that leads to aneuploidy. This demonstrates that, in a near-normal background, STK15/BTAK overexpression leads to centrosome amplification, chromosomal instability, and transformation in mammalian cells (Zhou *et al.*, 1998). Centrosomes ampli-

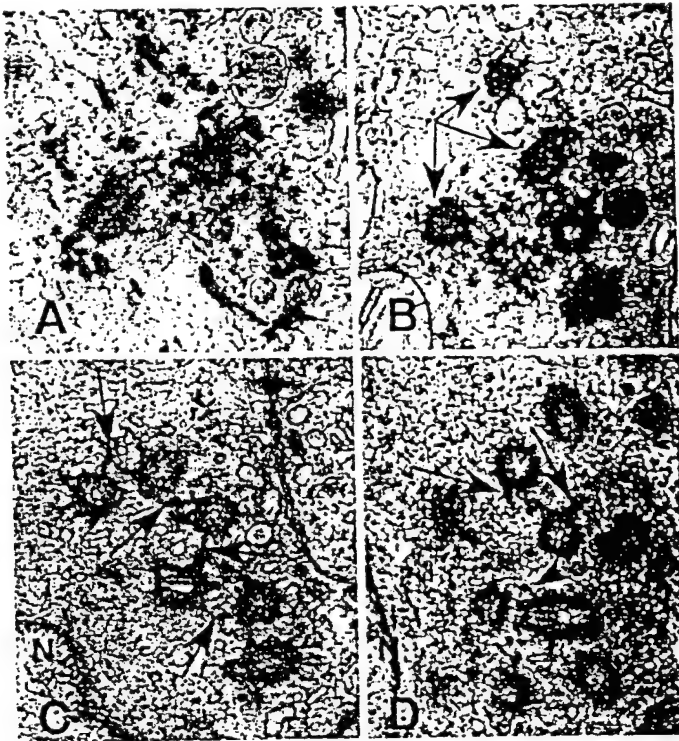
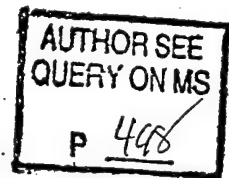


Figure 3 Transmission electron micrographs of amplified centrosomes in human breast tumors. (A) Excess pericentriolar material appears as darkly stained material appressed to the barrels of the centrioles and in small clumps next to the centrioles. Four centrioles are present in this thin section through an amplified centrosome. (B) One centriole encased in excess pericentriolar material and associated masses of pericentriolar material are present in the amplified centrosome. Centrosomes with excess pericentriolar material are associated with a high frequency of abnormal mitoses. (C, D) Clusters of six and nine centrioles linked together by fine fibers are present in these centrosomes. No excess pericentriolar material is present, and this type of centrosome is not associated with a high frequency of abnormal mitoses. (From Lingle and Salisbury, *American Journal of Pathology*, in press.)



fied by STK15/BTAK overexpression have not yet been ultrastructurally characterized; it will be of interest to learn if these centrosomes have excess pericentriolar material and/or other centrosome abnormalities seen in breast tumor centrosomes.

VI. Tumor Suppressor Proteins and the Centrosome

In the absence of tumor suppressor function through mutation, deletion, or disruption of the pathway in which they operate, cells are more likely to

undergo malignant transformation. Recently, two known tumor suppressor proteins, BRCA1 and p53, have been localized at the centrosome of mammalian cells (Brown *et al.*, 1994; Hsu and White, 1998), and it has been speculated that some of their tumor suppressor functions take place at the centrosome.

Germline mutations of the BRCA1 tumor suppressor gene predispose women to breast and ovarian cancers (Irminger-Finger *et al.*, 1999). BRCA1 protein is a large protein with numerous functional domains, including binding sites for p53, Rad51, RNA polymerase II holoenzyme, RNA helicase A, CtBP-interacting protein, c-myc, BRCA1-associated RING domain protein, and BRCA2 (Chen *et al.*, 1998, 1999; Irminger-Finger *et al.*, 1999). Immunofluorescence microscopy and analysis of isolated centrosomes provide evidence that BRCA1 protein is associated with centrosomes during mitosis. BRCA1 localizes with the centrosome during mitosis and coimmunoprecipitates with γ -tubulin, indicating that it may be involved with regulation of microtubule nucleation (Hsu and White, 1998). Mouse embryonic fibroblasts expressing only mutant BRCA1 with a targeted deletion of exon 11 contain multiple, functional centrosomes and undergo unequal chromosome segregation, abnormal nuclear division, and aneuploidy (Xu *et al.*, 1999). Xu and co-workers (1999) speculate that BRCA1 has an essential role in maintaining genetic stability through the regulation of centrosome duplication, and that the action of BRCA1 at the centrosome provide a molecular basis for the role of BRCA1 in tumorigenesis.

p53, the most frequently mutated gene in human cancers, is involved in checkpoint functions at the G1/S and the G2/M cell cycle transitions (Cross *et al.*, 1995; Hollstein *et al.*, 1998; Prives and Hall, 1999; Yin *et al.*, 1999). p53 mutation and nullizygosity are associated with increased chromosomal instability (Fukasawa *et al.*, 1997; Boyle *et al.*, 1998; Gualberto *et al.*, 1998; Weber *et al.*, 1998; Carroll *et al.*, 1999). Although most p53 is nuclear, a portion of p53 is localized at the centrosome in established human cell lines (Brown *et al.*, 1994) and in primary cultures of normal mammary epithelial cells (Lingle, unpublished). Mouse embryo fibroblasts (MEF) null for p53 undergo centrosome amplification (Fukasawa *et al.*, 1996), as do cells in tissues of mice nullizygous for p53 (Fukasawa *et al.*, 1997). Immunofluorescence labeling of p53 null MEFs with antibodies against γ -tubulin shows that these cells contain numerous centriole-sized spots arranged in clusters (Fukasawa *et al.*, 1996), similar to that seen in some human breast tumors (Lingle and Salisbury, 1999). Tissues of p53 null mice frequently are aneuploid and contain multipolar mitotic figures. *In vivo*, p53-independent apoptosis eliminates many of these aneuploid cells that contain amplified centrosomes (Fukasawa *et al.*, 1997).

In order to test the hypothesis that p53 mutation is the cause of centrosome amplification in human tumors, Weber and co-workers (1998) exam-

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ined three brain tumors for centrosome amplification and numerical chromosome aberration. Although the sample size was small—two neuroectodermal tumors with p53 mutations and one benign meningioma with wild-type p53 as a control tumor—the findings were consistent with the hypothesis. Centrosome amplification and evidence of multiple occurrences of chromosome segregation infidelity were found in the two tumors with mutant p53, whereas the control tumor had normal centrosomes and straightforward loss of three chromosomes. This is the first report correlating aneuploidy, centrosome amplification, and p53 mutation in human tumors.

Centrosome amplification also was associated with p53 mutation in breast carcinomas and squamous cell carcinomas of the head and neck (Carroll *et al.*, 1999). In this collection of tumors, however, not all tumors with amplified centrosomes had p53 mutations. Interestingly, those tumors with wild-type p53 and amplified centrosomes contained high levels of Mdm2. In normal cells, Mdm2 promotes rapid turnover of p53 by targeting it for ubiquitin-dependent degradation. Overexpression of Mdm2 causes an extremely short half-life of p53 and essentially makes the tumor cells behave as if they are p53 null, with attendant centrosome amplification and chromosome instability (Carroll *et al.*, 1999). Disruptions of other elements of the p53 pathway also have been shown to affect centrosome structure and function. p53-dependent induction of p21(cip-1/waf-1) is an important component of some cell cycle checkpoints. Reduced p21(cip-1/waf-1) expression results in gross nuclear abnormalities, centriole amplification, and polyploidy, most likely by uncoupling the centrosome cycle from the DNA cycle (Mantel *et al.*, 1999).

Although the most established function of p53 is as a tumor suppressor gene, certain mutations exhibit gain-of-function activities that increase oncogenic transformation through genomic instability (Gualberto *et al.*, 1998). Interestingly, the genomic instability occurs in the absence of transcriptional activation by p53; “thus p53 mutations can contribute to progression of a cancer cell not only by absence of p53 tumor suppressor activity but also by the presence of an activity that promotes genetic instability” (Gualberto *et al.*, 1998).

A similar gain-of-function was described in studies using a model of chemically induced papilloma in mice (Wang *et al.*, 1998). These studies used mice with wild-type p53 and knockout mice expressing no p53 or expressing mutant p53 under the control of a human keratin-1-based vector. The chemically induced tumors in mice with mutant p53 exhibited a less differentiated phenotype than those tumors elicited in p53 null mice or those in nontransgenic mice with wild-type p53. The p53 mutant tumors had a much higher frequency of centrosome anomalies than did p53 null or p53 wild-type tumors. The frequency of centrosome anomaly correlated

positively with metastasis and anaplasia (Wang *et al.*, 1998). The finding that centrosome anomalies were associated with less differentiated phenotypes and metastasis indicates that the interphase functions of the centrosome may be compromised in cells expressing mutant p53.

Fidelity in chromosome segregation and preservation of diploidy requires proper structure and function of the duplicated centrosomes; maintenance of cell and tissue polarity requires proper structure and function of interphase centrosomes. The evidence suggests that the centrosome is a nexus of regulation of cell cycle and cell polarity imposed in part by the actions of the p53 and BRCA1 tumor suppressor proteins.

VII. Maintenance of Cell and Tissue Polarity Minimizes Tumor Aggression in Model Systems

In mammary cell culture systems, the establishment of cell-cell contact and epithelial polarity is sufficient to stimulate the expression of β -casein (Roskelley *et al.*, 1994), while whey acidic protein additionally requires the formation of alveolar structures (Chen and Bissell, 1989; Lin *et al.*, 1995). In these cultured mammary epithelial cells, cell and tissue polarity regulates the expression of proteins associated with cell differentiation. Normal cell and tissue behavior is determined in part by interactions between the intermediate filament cytoskeleton and the extracellular matrix, and alterations in tissue structure can lead to the progression of tumors (Schmeichel *et al.*, 1998). Indeed, the malignant phenotype of cultured cells can be reverted to normal without changing the genotype of the cells. This is accomplished simply by application of a β 1-integrin inhibitory antibody to cells in the cell culture system (Weaver *et al.*, 1997). In response to application of the inhibitory antibody, cells essentially redifferentiate by forming three-dimensional acini with a basement membrane and reorganize cytoskeletons. Nude mice injected with antibody-treated tumor cells have tumors significantly reduced in number and size. Significantly, the observed phenotypes were reversible upon removal of the antibodies. These results show that in this model system the tissue phenotype (i.e., anaplastic vs. differentiated) is dominant over the cellular genotype (i.e., malignant vs. nontransformed) (Weaver *et al.*, 1997). Although not mentioned specifically by Bissell and co-workers, the centrosome, as the regulator of the microtubule cytoskeleton, is mechanically associated with the actin and intermediate cytoskeletons and the desmosomes that maintain tissue polarity. Perhaps disruptions of centrosome function that increase the anaplastic phenotype by adversely affecting tissue polarity are potentially correctable in the presence of malignant genetic lesions.

VIII. Conclusions

Centrosomes are involved with cancer in two possible ways. The first is through disruption of their function as the poles of the bipolar mitotic spindle apparatus. Centrosome defects that increase the chances of improper chromosome segregation during mitosis result in aneuploidy and lead to tumor progression. These defects include amplification of both the number and the size of centrosomes, hyperphosphorylation of centrosomal proteins, and an increase in their microtubule nucleating capacity (Lingle *et al.*, 1998; Pihan *et al.*, 1998; Weber *et al.*, 1998; Carroll *et al.*, 1999; Sato *et al.*, 1999). Centrosome-associated kinases and tumor suppressor proteins such as p53 and BRCA1 may be involved in regulating the transition of the centrosome from its interphase function to its mitotic function. Mutations in tumor suppressor genes and perturbations to the centrosome-associated kinase activities are likely causes of these centrosome defects that lead to aneuploidy. Recently, a century-old hypothesis first proposed by van Hanseman (1890, as cited in Rasnick and Duesberg, 1999), then by Boveri (1914), that aneuploidy is the cause of cancer has been resurrected (Brinkley & Goepfert, 1998; Rasnick and Duesberg, 1999). The hypothesis was refined to state that "cancer is the phenotype of cells above a certain threshold of aneuploidy" (Rasnick and Duesberg, 1999). It must be noted, however, that Duesberg suggests that centrosome abnormalities are a consequence of aneuploidy rather than aneuploidy being a consequence abnormal centrosomes (Duesberg, 1999).

Centrosomes also may be involved in cancer through their role in establishing and maintaining cell, and therefore tissue, polarity. Although the regulation of interphase functions of the centrosome is less well understood than are the mitotic functions, the centrosome and its interphase microtubule array are intimately involved with the actin and intermediate filament cytoskeletons. The three cytoskeletal systems act in concert to determine cell and tissue polarity. The fact that tissue phenotype (i.e., degree of polarity and differentiation) of cells cultured in a model system is dominant over the cellular genotype with regard to invasive potential (Weaver *et al.*, 1997) is a significant gain toward understanding malignant transformation in tumors.

Regardless of whether centrosome abnormalities are a cause or a consequence of cancer, the structure and function of the centrosome present promising opportunities for cancer therapy. A more thorough understanding of regulation of mitotic and interphase centrosome functions will facilitate exploitation of the centrosome as a target for effective therapeutic agents against cancer.

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